Iranian Journal of Veterinary Science and Technology
Vol. 6, No. 2, 2014, 42-52

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Tert-butylhydroquinone (TBHQ) improves antioxidant status in rat tissues following chronic diazinon intoxication

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Received: October 8, 2014 Accepted: January 17, 2015

Abstract

Considering that the involvement of oxidative stress has been implicated in the toxicity of organophosphate insecticides, the objective of the present study was to investigate antioxidants and oxidative stress markers in the liver and kidney of Wistar rats treated with chronic doses of diazinon. In addition, the effect of Tert-butylhydroquinone (TBHQ), a food-additive antioxidant, on attenuation of diazinon-induced oxidative stress was evaluated. 28 rats were randomly assigned to 1 of 4 treatment groups: diazinon (10 mg/kg BW, once a day; n=7), TBHQ (0.028 g/kg of diet, once a day; n=7), TBHQ + diazinon (diazinon; 10 mg/kg BW, once a day + TBHQ; 0.028 g/kg of diet, once a day; n=7) and control (corn oil, as vehicle of diazinon and TBHQ, n=7). TBHQ, diazinon and corn oil were given to rats orally via gavage for 7 weeks. Total thiol groups, ferric reducing antioxidant power (FRAP) and malondialdehyde (MDA) levels in liver and kidney tissues were investigated at the end of 7th week. Treatment with diazinon significantly increased MDA level, as a marker of lipid peroxidation, in the kidney ($p<0.05$). Co-administration of TBHQ with diazinon significantly increased total thiol groups and FRAP levels in liver and kidney ($p<0.05$). However, TBHQ did not reduce lipid peroxidation in the tissues. The results of the present study showed that administration of TBHQ at a dose of 0.028 g/kg of diet, increased antioxidant capacity of tissues of rats treated with diazinon, though this increase was not sufficiently effective to reduce lipid peroxidation caused by diazinon. Higher amounts of TBHQ might be more effective in attenuation of diazinon-induced lipid peroxidation.

Keywords: diazinon, oxidative stress, TBHQ, rat

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Introduction

Organophosphate compounds (OPs) are commonly used as insecticides, and are generally the most toxic of all pesticides to vertebrate animals (Dawood Shah and Iqbal, 2010). It is well established that OPs cause inhibition of acetylcholinesterase activity in the target tissues (Buyukokuroglu *et al.*, 2008). However, the toxic effect of OPs is not limited to inhibition of cholinesterase. It has recently been reported that oxidative stress may be an important factor in OP-induced toxicity (Lu *et al.*, 2012). The imbalance between generation of reactive oxygen species (ROS) and antioxidant mechanisms in the body is called oxidative stress, which has important health implications (Heidarpour *et al.*, 2012). ROS are produced as the result of the metabolism of OPs by cytochrome P450s. The disturbance in the cell redox system is another mechanism implicated in the generation of ROS in OPs exposure. In addition, the exposure to OPs induces hyperglycemia leading to increase of non-enzymatic glycation by the binding of glucose or by-products to proteins and formation of advanced glycation end products. Glycated proteins activate specific membrane receptors and induce an intracellular oxidative stress (Lukaszewicz-Hussain, 2010). ROS can be detoxified by enzymatic and non-enzymatic antioxidants, which are essential for the conversion of ROS to harmless metabolites as well as to protect and restore normal cellular metabolism and functions (El-Shenawy *et al.*, 2010). OPs pesticides have been reported to decrease the level of antioxidants (Muniz *et al.*, 2008). Lipid peroxidation is a complex process resulting from ROS reactions in biological membranes, which are rich in polyunsaturated fatty acids (Oruc and Usta, 2007).One of the molecular mechanisms of the toxicity of some pesticides seems to be lipid peroxidation (Mansour and Mossa, 2009).

Diazinon (o,o-diethyl-o-[2-isopropyl-6-methyl-4- pyrimidinyl]phosphorothioate) is one of the most commonly used OPs in the world (Kalender *et al.*, 2005). Diazinon causes changes in liver enzymes and biochemical indices and affects mitochondrial membrane transportation and cytochrome P450 system in hepatocytes (Nakagawa and Moore, 1999; Sams *et al.*, 2003). In addition, it has also been shown that diazinon caused an increase in lipid peroxidation in rat erythrocytes (Sutcu *et al.*, 2007). Treatment of rats with diazinon eventuated in decreased renal antioxidants and enhanced lipid peroxidation with concomitant renal damage, which are involved in the diazinon-induced renal oxidative stress and toxicity (Dawood Shah and Iqbal, 2010).

It has been shown that antioxidant substances protect cells against deleterious effects of environmental agents. In recent years, some agents with antioxidant effects (e.g. vitamins C and E, N-Acetylcysteine and zinc) have been used to decrease cellular oxidative stress, and thus cellular damage, in cases of organophosphate poisoning (El-Shenawy *et al.*, 2010; Sameeh *et al.*, 2009; Sutcu *et al.*, 2007; Ogutcu *et al.*, 2006; Cankayali, *et al.*, 2005). *Tert*-butylhydroquinone (TBHQ) is one of the few antioxidants permitted for use in foods. TBHQ has been shown to induce nuclear translocation of Nrf2 that regulates antioxidant genes as an adaptive response to oxidative stress (Tasset *et al.*, 2010).

Little attention has been given to the chronic low dose effects of pesticides, which may not have clinically recognizable symptoms but could produce subtle cumulative metabolic effects that eventually affect the overall health of an animal. In addition, there is no study about the ameliorative effect of TBHQ on oxidative stress induced by diazinon. Therefore, the present study was undertaken to evaluate the possible protective effect of TBHQ on oxidative stress and antioxidant status after 7 weeks exposure to sub-lethal dose of diazinon

in rats.

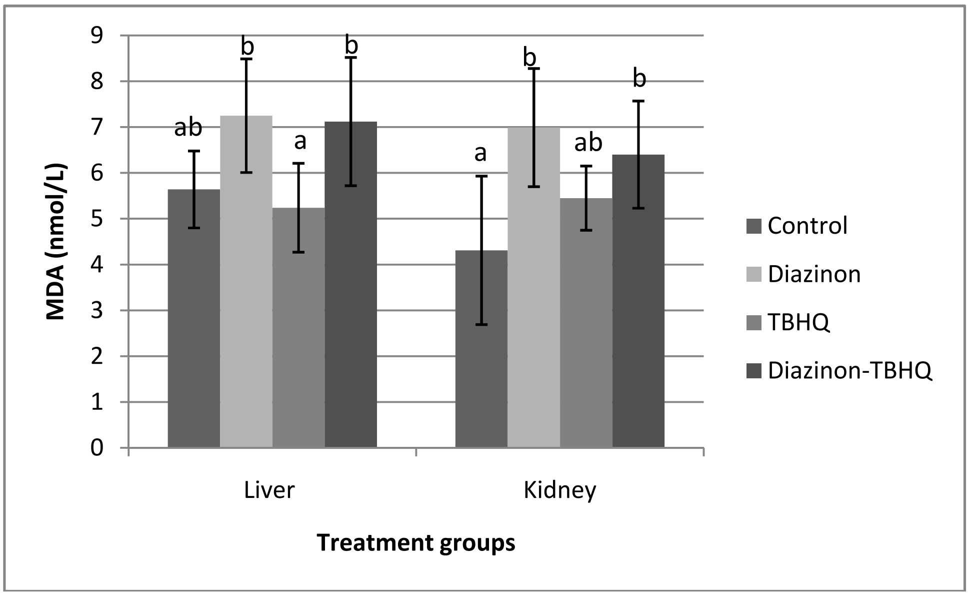


Figure 1. Effects of diazinon and TBHQ on the MDA level in liver and kidney of rats.
Values are expressed as mean ± SD. Means lacking a common lowercase letter differ (*p*<0.05).

Materials and methods

Chemicals

TBHQ, diazinon, tris, potassium chloride (KCl), thiobarbituric acid (TBA), pyridine, n-butanol, sodium hydroxide (NaOH), 5-5'-dithio-bis-2-nitrobenzoic acid (DTNB), ethylenediaminetetraacetic acid (EDTA), sodium acetate trihydrate, glacial acetic acid, 2,4,6-tripyridyl-s-triazine (TPTZ), hydrochloric acid (HCI), $FeCl_3$. $6H_20$ and $FeSO_4.7H_20$ of technical grade used in this study were supplied by Sigma-Aldrich (Germany) or Merk (Germany) companies.

Animals

Male Wistar-rats (weighing approximately 230–250 g) were purchased from the Razi Vaccine and Serum Research institute, Mashhad, Iran. All these animals were acclimatized for one week before the onset of experiment. The animals were housed in plastic (polypropylene) cages using paddy husk bedding at room temperature (25 ± 1°C) in a 12-hour light/dark cycle with 50 ± 5% humidity. The animals had free access to commercial pellet diet (Javaneh Khorasan, Mashhad, Iran) and water ad libitum. The experiment was approved by the Animal Welfare Committee of the School of the Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

Experimental protocol

Twenty eight adult male Wistar-rats divided randomly into four groups having seven animals in each. The compounds were administrated in the morning (between 9:00 and 11:00 AM) to non-fasted rats. All rats were treated for 7 weeks.

Group 1: control group

The control group received corn oil (vehicle of diazinon and TBHQ) through gavages once a day.

Group 2: diazinon-treated group

Diazinon at a dose of 10 mg/kg BW/day in corn oil was given through gavage to rats once a day. The selection of dose regimen was based on previously published studies which indicate substantial alterations in many of the biochemical parameters at this dose (Dawood Shah and Iqbal, 2010; Kalender *et al.*, 2005).

Group 3: TBHQ treated group

The dose of TBHQ was selected based on

previously published studies which indicate anti-oxidative effects of TBHQ at this level (0.028 g/kg of diet; Nishizono *et al.*, 2000). For this purpose, the food intake of rats was measured during the acclimatization period. Pre-weighed food was provided in standard stainless steel hoppers. After 24 h, the amount of food remaining, including any on the bottom of the cages or any that had spilled onto plastic sheets placed under each cage was recorded. Intake was calculated as the weight (in grams) of food provided minus that recovered. Based on recommendations for the most effective dose of TBHQ (0.028 g/kg of diet) and registered food intake, the final concentration of TBHQ was calculated and was given through gavage to rats once a day.

Group 4: TBHQ + diazinon-treated group

TBHQ and diazinon (at above-mentioned doses) were administered orally via gavage needle.

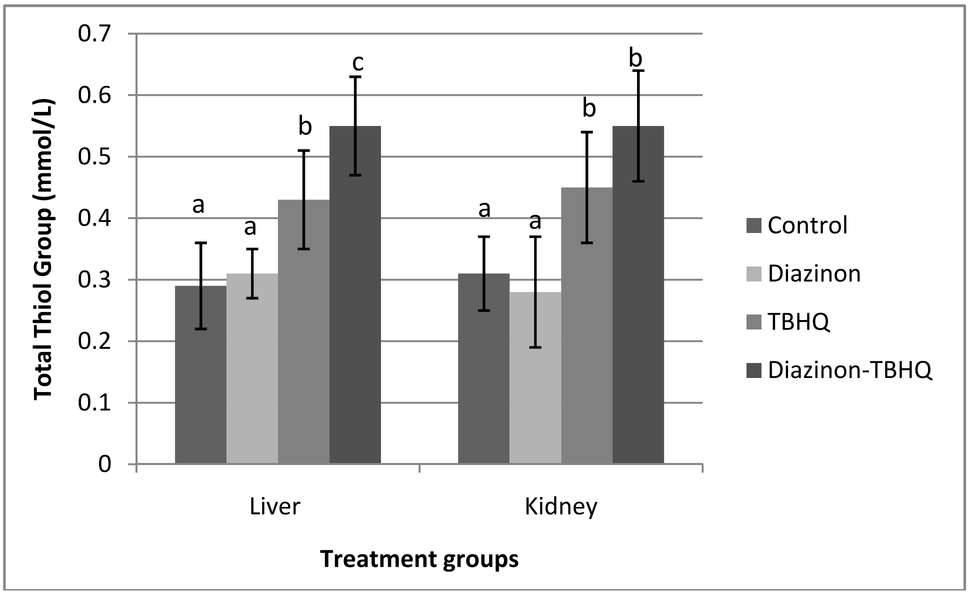


Figure 2. Effects of diazinon and TBHQ on the total thiol group level in liver and kidney of rats. Values are expressed as mean ± SD. Means lacking a common lowercase letter differ (*p*<0.05).

Tissue preparation

The animals were euthanized by CO_2 24 h after the last dose of diazinon or TBHQ. Liver and kidney tissues of these animals were taken quickly, cleaned free of extraneous material and perfused immediately with sodium phosphate buffer (pH 7.4). Tissue samples were minced, cut into small pieces and then dried on a filter paper and homogenized (10% w/v) in ice-cold 1.15% KCl-0.01 M sodium, potassium phosphate buffer (pH 7.4) by ‘‘Silent crusher M’’ type homogenizer (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). The homogenate was centrifuged at 18,000g for 20 min at 4^oC, and the resultant supernatant was used for the determination of oxidative stress markers.

Lipid peroxidation assay

The extent of lipid peroxidation was estimated as the concentration of thiobarbituric acid reactive product malondialdehyde (MDA) by using the method of Placer *et al.* (1966). The reaction mixture consisted of 0.2 ml of homogenized tissue, 1.3 ml of 0.2 M Tris–0.16 M KCl buffer (pH 7.4) and 1.5 ml of thiobarbituric acid reagent. The mixture was heated in a boiling water bath for 10 min. After cooling, 3 ml of pyridine/n-butanol (3:1, v/v) and 1 ml of 1 N sodium hydroxide were added and mixed by vigorous shaking. A blank

was run simultaneously by incorporating 0.2 ml distilled water instead of the homogenized tissue. The absorbance of the test sample was read at 548 nm. The nmol of MDA per ml was calculated using 1.56×10^5 as extinction coefficient.

Total thiol groups assay

Total thiol groups of homogenized tissues were measured spectrophotometrically at 412 nm using DTNB as the reagent (Hu and Dillard, 1994). After adding tris buffer to homogenized tissue, first absorbance was read at 412 nm (A1). Then DTNB was added and second absorbance

at 412 nm was done (A2). The concentration of total thiol groups was calculated and expressed as mmol/l.

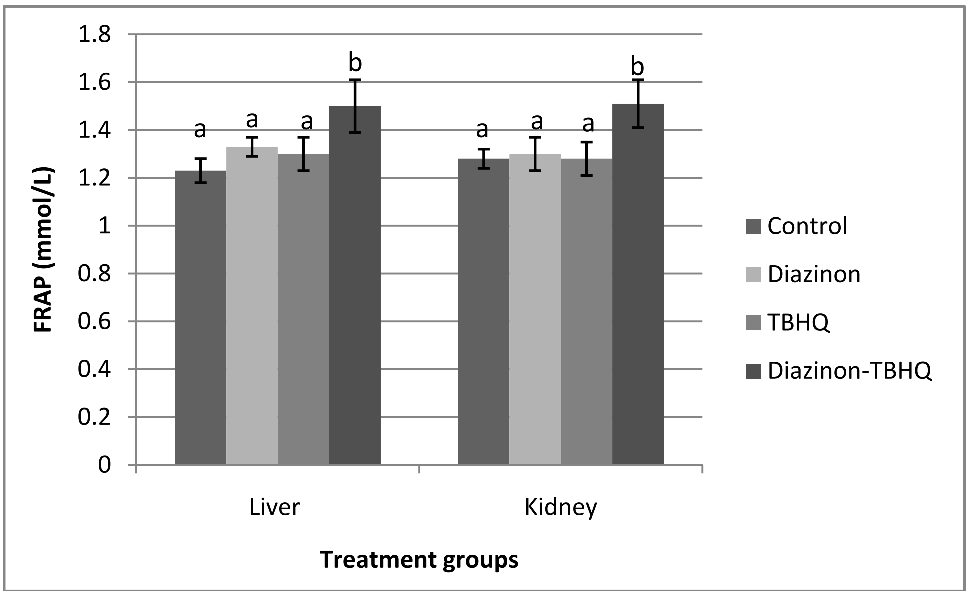


Figure 3. Effects of diazinon and TBHQ on the FRAP level in liver and kidney of rats.
Values are expressed as mean ± SD. Means lacking a common lowercase letter differ (p<0.05).

Ferric reducing antioxidant power (FRAP) assay

The total antioxidant capacity of the homogenized tissues was measured using FRAP assay, which depends upon the reduction of ferric tripyridyltriazine (Fe(III)-TPTZ) complex to the ferrous tripyridyltriazine (Fe(II)-TPTZ) by a reductant at low pH. (Fe(II)-TPTZ has an intensive blue color and can be monitored at 593 nm (Benzie and Strain, 1996).

Statistical analysis

Statistical analysis was conducted using SPSS for windows (release 16, SPSS Inc., Chicago, IL) with a p value of <0.05 as statistically significant. Data were expressed as mean±standard deviation (SD). One way ANOVA was used to compare means among the different groups. Following analysis of variance, significant between-group differences were detected by the bonferroni test.

Results

A significant increase in MDA levels (p<0.05) in the kidney tissue was evident in the diazinon group, when compared to the control group. However, no statistically significant differences were detected for liver MDA between diazinon and control groups. TBHQ alone or associated with diazinon, did not reduce lipid peroxidation in the evaluated tissues and no significant differences were observed for MDA level between TBHQ and

control groups and between diazinon and diazinon-TBHQ groups (fig.1).

Treatment with diazinon did not induce any significant change in the thiol group and FRAP levels of liver and kidney tissues. TBHQ alone or associated with diazinon, significantly increased thiol groups. Rats of the TBHQ and diazinon-TBHQ groups presented a significant increase in thiol groups ($p<0.05$), when compared to control and diazinon groups (fig.2). Co-administration of TBHQ with diazinon increased FRAP level in the liver and kidney tissues ($p<0.05$), when compared with the diazinon group (fig.3).

Discussion

Extensive application of OPs is usually accompanied with serious problems of pollution and health hazards. OPs act as pro-oxidants and elicit oxidative effects in multiple organs (Limon-Pacheco and Gonsebatt, 2009). Bagchi *et al.* (1995) found that OPs may induce in vitro and in vivo generation of ROS, such as hydrogen peroxide, superoxide and the hydroxyl radical. Oxidative stress has been described in acute and chronic exposure to OPs in both animals and humans (El-Shenawy *et al.*, 2010; Agrawal and Sharma, 2010; Eduardo *et al.*, 2006; Mashali *et al.*, 2005; Kovacic, 2003; Zhou, *et al.*, 2002; Banerjee *et al.*, 2001). The present study was designed to elucidate the potential antioxidant effects of tBHQ, which is used as a food-additive antioxidant for human.

In the present study, diazinon promoted lipid peroxidation in the kidney tissue of rats treated with 10 mg/kg of diazinon for 7 weeks. Lipid peroxidation causes profound alterations in the structure and functions of the cell membrane including decreased membrane fluidity and increased membrane permeability (Ojha *et al.*, 2011). Lipid peroxidation results in the disarrangement and ultimately, disruption of cell membranes, which leads to cell death (Oruc and Usta, 2007). It has been shown that OPs caused increase of lipid peroxidation through their interference with membrane dependent processes (Kalender *et al.*, 2007, 2010; Elhalwagy *et al.*, 2008; Uzun *et al.*, 2010; El-Demerdash, 2011). In consistent with the present study, Dawood Shah and Iqbal (2010) reported that treatment of rats with diazinon significantly enhanced renal lipid peroxidation in a dose-dependent manner, reflecting the formation of ROS in rat kidney. At the lower dose of diazinon 10 mg/kg body weight, about 1.1-fold increase in lipid peroxidation occurred compared to control. However, at the higher dose of diazinon 30 mg/kg body weight, the increase in lipid peroxidation was around 1.3-fold (Dawood Shah and Iqbal, 2010). In addition, enhanced lipid peroxidation has been observed following diazinon administration in erythrocytes (Altuntas, *et al.*, 2004), liver (Amirkabirian *et al.*, 2007) brain (Jafari *et al.*, 2012), spleen (Jafari *et al.*, 2012) and pancreas (Gökalp *et al.*, 2005) of rats. High lipid peroxidation may be due to oxidation of molecular oxygen to produce superoxide radicals. This reaction is also the source of H_2O_2, which causes the production of MDA by initiating the peroxidation of unsaturated fatty acids in the membrane (Uner *et al.*, 2001). The difference of results in different studies is caused by the dose, exposure time, and administration route of toxin. The observed differences in diazinon-induced lipid peroxidation among various tissues may depend on several factors such as oxygen consumption, metabolic activity rate, susceptibility to oxidants and many more (Jafari *et al.*, 2012).

TBHQ, one of the most powerful synthetic antioxidants, is recommended by American Institute of Nutrition (AIN) as a supplement to the rodent diet (Philip *et al.*, 1993). Anti-oxidative effect of TBHQ has been reported in experimental studies. TBHQ reduced the level of lipid peroxidation in mice with traumatic brain injury (Lu *et al.*, 2014). It protects the living animal and cell lines against acute

toxicity and oxidative insult (Lu *et al.*, 2014). TBHQ is able to induce the nuclear translocation of transcription factor NF-E2-related factor 2 (Nrf2), which in turn regulates the expression of vitagenes codifying for cytoprotective phase 2 antioxidant proteins, such as glutathione- S-transferase, NAD(P)H quinone oxidoreductase and heme-oxygenase-1 (Tasset *et al.*, 2010). In the present study, Co-administration of TBHQ with diazinon significantly increased total thiol groups and FRAP levels in liver and kidney tissues. In agreement with the present study, Lu *et al.* (2014) reported that pretreatment with TBHQ significantly increased antioxidant activity in mice with traumatic brain injury. Although TBHQ increased antioxidant levels in rats receiving diazinon, it could not attenuate lipid peroxidation in them.

While diazinon administration showed no significant alterations on antioxidant levels, combined TBHQ and diozinon administration increased antioxidant level in liver and kidney tissues. The observed results could be explained by increased challenge with free radicals and oxidants in the diazinon-received rats. In the TBHQ + diazinon group, elevated free radical generation could be encountered with increased antioxidant capacity via TBHQ administration. The elevated levels of measured antioxidants are due to the adaptive response to the generated free radicals. The increase in antioxidants may be explained by the fact that oxidants activate gene expression through antioxidant responsive elements (Rushmore *et al.*, 1991). However, the total antioxidant defense mechanism was not able to protect the tissues from oxidative damage caused by diazinon, as evidenced by lipid peroxidation.

The present study is the first investigation in which protective effects of TBHQ against diazinon-induced oxidative stress was evaluated. Several limitations of the present study should be considered when interpreting the results. The used amount of TBHQ in the present study was selected based on recommendations in the AIN-93G formula (Philip *et al.*, 1993). Higher amounts of TBHQ might be more effective in attenuation of diazinon-induced lipid peroxidation.

Acknowledgements

This study was supported by research fund of Ferdowsi University of Mashhad (project no. 3/29214). The authors wish to thank technicians who kindly helped us for sample collection of this study.

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Iranian Journal of Veterinary Science and Technology
Vol. 6, No. 2, 2014, 42-52

IJVST

اثرات محافظتی ترت بوتیل هیدرو کوئینون (TBHQ) در مقابل استرس اکسیداتیو ناشی از دیازینون در رت

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دریافت مقاله : ۱۳۹۳/۰۷/۱۶ پذیرش نهایی: ۱۳۹۳/۱۰/۲۷

چکیده

با توجه به نقش استرس اکسیداتیو در بیماری زایی سموم ارگانوفسفره، در مطالعه حاضر اثرات مسمومیت مزمن با دیازینون بر شاخص های استرس اکسیداتیو بافت های کبد و کلیه رت ویستار مورد ارزیابی قرار گرفت. به علاوه اثرات محافظتی ترت بوتیل هیدرو کوئینون (TBHQ؛ به عنوان یک آنتی اکسیدان غذایی قوی) در جلوگیری از بروز آسیب اکسیداتیو ناشی از دیازینون در این بافت ها بررسی گردید. گروه های مورد مطالعه شامل ۴ گروه هر کدام به تعداد ۷ سر رت بود: گروه کنترل (دریافت کننده روغن ذرت به صورت روزانه)، گروه دیازینون (۱۰ میلی گرم به ازای هر کیلوگرم وزن بدن حل شده در روغن ذرت)، گروه TBHQ (۰/۰۲۸ گرم به ازای هر کیلوگرم ماده غذایی، حل شده در روغن ذرت) و گروه دیازینون– TBHQ (سم و آنتی اکسیدان با دوزهای ذکر شده). خوراندن روغن، سم یا آنتی اکسیدان به مدت ۷ هفته و بصورت روزانه توسط لوله گاواژ صورت گرفت. پس از پایان مطالعه شاخص های استرس اکسیداتیو شامل مالون دی آلدهید، گروه تیول و ظرفیت تام آنتی اکسیدانی (FRAP) در هموژنیزه بافت های کبد و کلیه اندازه گیری شد. تجویز دیازینون موجب افزایش معنی دار (p<0.05) میزان مالون دی آلدهید به عنوان شاخص پراکسیداسیون لیپیدی، در بافت کلیه گردید. تجویز همزمان TBHQ با دیازینون، مقدار گروه تام تیول و FRAP را در بافت های کبد و کلیه بصورت معنی داری افزایش داد (p<0.05)، اما اثری در مقدار مالون دی آلدهید بافت‌ها نداشت. مقدار تجویز شده از TBHQ با وجود فعالیت آنتی اکسیدانی و افزایش معنی دار گروه تیول و ظرفیت تام آنتی اکسیدانی، به طور کارامدی قادر به بهبود وضعیت پراکسیداسیون لیپیدی ایجاد شده توسط دیازینون در بافت های مختلف نبود. به نظر می رسد استفاده از مقادیر بالاتر TBHQ برای مهار پراکسیداسیون لیپیدی ناشی از مسمومیت مزمن دیازینون ضروری باشد.

واژگان کلیدی : دیازینون، استرس اکسیداتیو، ترت بوتیل هیدرو کوئینون، رت